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Mussels do not directly assimilate fish farm wastes: Shifting the rationale of Integrated Multitrophic Aquaculture to a broader scale

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Summary

Pollution is one of the most significant issues that is currently impeding the development of fish farming. Integrated multi-trophic aquaculture (IMTA) has the potential to reduce the accumulation of organic wastes in the environment by using taxa of lower trophic levels such as filter feeders. However, the capacity of filter feeders to assimilate significant quantities of fish farm wastes has not yet been fully tested *in situ*. We analyzed the stable isotopes $\delta^{13}C$ and $\delta^{15}N$ in mussels from six fish farms and from six other areas that were not influenced by fish farming, at two water strata (surface and mid-water) across a marked gradient of eutrophication along more than 900 km of coastline in the Western Mediterranean. We found that the mussels did not directly assimilate fish farming wastes. Consequently, fish farming wastes did not constitute a major component of mussel diet, irrespective of local productivity and depth in the water column. These outcomes do not necessarily mean that IMTA is not suitable in other cases, but rather that there should be a shift in the rationale of IMTA by modifying the concept of direct assimilation of wastes to a more general approach of IMTA based on regional budgets of nutrients.

Capsule: Mussels do not directly assimilate fish farming wastes. Consequently, there should be a shift in the rationale of IMTA based on regional budgets of nutrients.

Keywords: Coastal pollution, fish farming, IMTA, Mediterranean, *Mytilus galloprovincialis*, stable isotopes.

Introduction

The importance of aquaculture for food production is growing at an ever increasing rate, and this trend is expected to continue in the coming decades. Marine environments are particularly important in this regard, as the space is much less restricted compared to other food production activities that take place entirely on land (FAO 2014). However, the rate of establishment of aquaculture is impeded by the impacts of pollution that may generate. In particular, fish farming generates a large quantity of organic wastes, mainly derived from feeding, in the form of uneaten feed and fish faeces (Sanz-Lazaro & Marin 2008). Uneaten feed and fish faeces consist of large and small particles. Large faecal particles and uneaten feed sink rapidly and may accumulate in sediments on the seafloor where they may be consumed by detritus-eating animals. Small particles of waste can remain in suspension and then be consumed by filter-feeding zooplankton or by visual feeders, such as fish, in the water column, or by mussels. When accumulate on the seabed, these wastes lead to oxygen depletion and the prevalence of anaerobic metabolic pathways, deteriorating of the ecological status of the benthic system and consequently on its ecological functions (Holmer, Wildish & Hargrave 2005; Karakassis *et al.* 1999; Sanz-Lazaro & Marin 2011).

Under this scenario, integrated multi-trophic aquaculture (IMTA) has emerged as a potential tool to help fish farming become a more environmentally friendly activity, by culturing combinations of species at different trophic levels (Neori *et al.* 2004). The purpose of IMTA is two-fold, seeking to reduce environmental impacts while increasing production. It aims to limit the wastes derived from aquaculture by culturing species with a low trophic level that can feed on the wastes generated by cultured species at higher trophic levels. This approach is expected to maximize the production of the low-

trophic-level species due to an increase on the availability of food derived from the release of wastes by high-trophic-level species (Soto 2009).

IMTA has been designed using generally fish as the high-trophic-level species, and, usually, extractive and filter-feeding species as the low-trophic-level species, for which mussels and oysters are the main groups used in marine IMTA (Cranford, Reid & Robinson 2013). The concept of IMTA is appealing and promising. Bivalves have the capacity to accumulate fish farm wastes (Handa *et al.* 2012b; MacDonald, Robinson & Barrington 2011; Redmond *et al.* 2010): models simulating bivalve production predict significantly greater yields when cultured under IMTA compared to monocultures (Ferreira, Saurel & Ferreira 2012; Sara *et al.* 2012). Nevertheless, the implementation of IMTA using fish and bivalves has yielded contrasting results. In some cases bivalves have had a higher growth rate close to fish farms (Jones & Iwama 1991; Wallace 1980), but in other cases the fish farms did not seem to influence their growth (Cheshuk, Purser & Quintana 2003; Navarrete-Mier, Sanz-Lazaro & Marin 2010; Peharda *et al.* 2007), or only had an influence at certain times of the year (Handa *et al.* 2012a). Nevertheless, the observed changes in growth rate do not necessarily prove the assimilation of fish farming wastes by bivalves.

Isotopes have been widely used in ecology to decipher trophic pathways, allowing measurements of time-integrated assimilation of foods (Hobson & Welch 1992) and differentiation in the origin (terrestrial or marine) of food sources (Darimont, Paquet & Reimchen 2009). Isotopes have previously been used to trace fish farm wastes (Holmer *et al.* 2007; Sara *et al.* 2004), and are suitable to test the assimilation of fish farming wastes (Sanz-Lazaro *et al.* 2015; Vizzini & Mazzola 2004; Yokoyama, Tadokoro & Miura 2015). They can be distinguished from other sources of marine food because a considerable part of the fish food ingredients have a terrestrial origin, which

is $\delta^{13}C$ depleted (Ytrestol, Aas & Asgard 2015). As a result, this isotope has been used to evaluate the assimilation of fish farming wastes in different organisms and communities (Dolenec *et al.* 2006; Irisarri *et al.* 2015; Navarrete-Mier, Sanz-Lazaro & Marin 2010; Sanz-Lazaro *et al.* 2011).

Filter-feeding bivalves have the capacity to assimilate fish farming wastes under laboratory conditions (Handa *et al.* 2012b; Reid *et al.* 2010), although *in situ* pilot studies have shown that fish farming wastes do not make up a substantial part of their diet (Handa *et al.* 2012a; Irisarri *et al.* 2015; Navarrete-Mier, Sanz-Lazaro & Marin 2010). This may be because IMTA biomitigation capacity may depend on the trophic state of the water column and/or benthos (Cranford, Reid & Robinson 2013). Thus, the suitability of IMTA is expected to be greater in areas with naturally-low nutrient concentration, as these areas have low densities of plankton with low food quality in comparison to more-eutrophic areas (Both, Parrish & Penney 2012; Lander *et al.* 2013; Troell *et al.* 2003).

Previous *in situ* studies have been constrained by the specific environmental characteristics of the single locations at which each experiment was carried out, preventing the extrapolation of the results to other areas. In addition, water depth is expected to be a key parameter in the growth of filter-feeding bilvalves (Fuentes *et al.* 2000), particularly in IMTA (Mazzola, Favaloro & Sara 1999), for which the availability of particulate fish farming wastes may be markedly stratified across the water column. However, the importance of depth has not generally been considered in the implementation of IMTA (but see Filgueira *et al.* 2017). Hence, there is an urgent need for a comprehensive assessment of the feasibility of IMTA systems. This would help to optimize the use of this promising tool to diminish the environmental impact of fish farming, and thus to remove this impediment to the expansion of aquaculture.

The aim of this study is to test whether filter feeders are able to use fish farming wastes as trophic resources in coastal areas close to fish farms, based on the IMTA rationale. We hypothesize that the direct assimilation of organic wastes from fish farming may be related to the natural productivity of the water body, with increased assimilation when primary production is low. To investigate this, we tested whether mussels (*Mytilus galloprovincialis*) could ingest a significant proportion of organic wastes derived from fish farming under different levels of eutrophication and depths in the water column. We analyzed the content of two stable isotopes - $\delta^{13}C$ and $\delta^{15}N$ - in mussels from six fish farms and from six other areas that were not influenced by fish farming. Samples were taken at two water strata - surface (from 3 to 5 m depth) and mid-water (from 12 to 16 m depth) - covering a marked gradient of eutrophication along the Western Mediterranean coast.

Material and methods

Study area

Mussels were taken from the Spanish coast along the Western end of the Mediterranean, covering more than 900 km of coastline from the southern limits of the Balearic Sea to the upwelling of the Alborán Sea (Fig. 1; Table S1). Although the Mediterranean is generally oligotrophic, the study area has a marked eutrophication gradient ranging from 0.32 to 3.5 mg chl *a* m^{-3} $year^{-1}$ (see Fig. S1; D'Ortenzio & D'Alcala 2009).

Sampling

To ensure that mussels had enough time to accumulate the isotopic signal we only analyzed mussels that were longer than 45 mm, as the growth rates of *M. galloprovincialis* in the Mediterranean are below 50 mm per year (Abada-Boudjema &

Dauvin 1995; Ceccherelli & Rossi 1984). Mussels were taken from 12 locations during late summer and autumn of 2015. Current from two oceanographic bouys, Cabo de Gata and Cabo de Palos, showed that for this period of time the current speed was 22.8 and 25.9 $cm \cdot s^{-1}$, respectively and the dominant current was in both cases SW (Puertos del Estado 2017). Six were at fish farms (labelled with "F" and the corresponding number from 1 to 6) culturing gilthead sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*), which were fed with pellets of fish feed (mainly composed of plant protein and oil, as well as, marine protein and oil; Fernandez-Jover *et al.* 2011; Ytrestol, Aas & Asgard 2015) at an annual rate of 300 to 1,000 tonnes per year depending on the size of the fish farm. The other six locations were reference sites that were considered unlikely to be influenced by fish farming (labelled with "R" and the corresponding number from 1 to 6); they were generally more than 10 km from the closest fish farm or any other possible source of anthropogenic activity that produced organic wastes. Due to the difficulty of locating structures that supported mussels, reference location R5 was located less than 3 km from fish farm F4. However, R5 was located “upstream” of the main current of the area that passed site F4 (Sanz-Lazaro *et al.* 2011), and fish farming wastes at the time of highest production do not reach more than 350 m "downstream" of the main current (Sanz-Lazaro, Navarrete-Mier & Marin 2011). In addition, because of the aforementioned difficulty in finding references sites, two of the reference sites were located 7 km away from each other (Fig. 1; table S1).

In the fish farm facilities, mussels were taken from ropes and cage structures. In the reference locations we searched buoys of various types, primarily those that delimited marine protected areas. At each location mussels were taken by scuba divers at two water strata, surface (from 3 to 5 m depth) and mid-water (from 12 to 16 m depth). Mussels were collected during the second half of 2014. The distance from each

location to the coast was always greater than 200 m in order to avoid possible interferences in the $\delta^{13}C$ signature between fish feed and other terrestrial sources. In addition to the mussels, two types of fish food used in the fish farms were also analyzed.

Sample processing

Mussels were chilled in a portable cooler immediately after collection, and after arriving on land they were immediately frozen and transported frozen to the laboratory where they were stored at -20ºC. Mussels were then thawed, opened and dissected to remove (and discard) the digestive system. The tissues used for analysis were the mantle, pedal sinus, adductor muscle and gills. They were rinsed with distilled water, lyophilized, ground to a powder and stored at −20 °C prior to analysis (Riera & Richard 1996). Fish feed was also lyophilized, ground to a powder and stored at−20 °C.

Sample analysis

The $\delta^{13}C$ and $\delta^{15}N$ ratios of the samples were measured using an elemental analyzer (ThermoFinnigan Flash EA 1112, Thermo Electron, Bremen, Germany) connected to a mass spectrometer of isotopic relationships (Delta Plus, Thermo Finnigan, Bremen, Germany).

The isotopic ratio data was reported as follows:

$$\delta^{13}C \text{ or } \delta^{15}N = (Rsample / Rstandar - 1)\ 1000\ (‰)$$

where R represents the 13C/12C or 15N/14N ratio for $\delta^{13}C$ and $\delta^{15}N$, respectively.

$\delta^{13}C$ values were reported as the relative deviation from the Vienna Pee Dee Belemnite Limestone Standart (v-PDB), while $\delta^{15}N$ results were reported as the relative deviation from atmospheric nitrogen.

Data analysis

$\delta^{13}C$ and $\delta^{15}N$ content data were analyzed using an ANOVA with three factors. The first factor was *aquaculture influence*, fixed and orthogonal, with two levels, with and without influence. The second was *depth*, fixed and orthogonal, with two levels, surface and mid-water. The third was *location*, random and nested in *aquaculture influence*. The experimental unit was a single mussel, and five replicates were taken at each level.

In addition, we used the $\delta^{13}C$ and $\delta^{15}N$ data to calculate the metrics described by Layman et al. (2007) for each five individuals taken at each location and depth, which allowed us to quantitatively characterize and compare trophic parameters among mussel populations *sensu* Darimont et al. (2009). The metrics used were: 1) $\delta^{13}C$ range (CR), which indicates the quantity of basal resources and niche diversification at the base of the food web, 2) $\delta^{15}N$ range (NR), which shows the degree of trophic diversity, 3) mean distance to centroid (CD), which shows the overall degree of trophic diversity, and is particularly useful in cases with outlier species, 4) mean nearest neighbour distance (NND), which indicates dietary variation among individuals and 5) standard deviation of the nearest neighbour distance (SDNND), which indicates the evenness of the distribution of trophic niches in a population. For a more thorough description of the metrics and the algorithms see Layman et al. (2007). With these metrics we performed a two-way factorial ANOVA taking each population as replicates, and consequently *aquaculture influence* and *depth* as factors, to test if there were differences in the

trophic parameters of mussels due to the allochthonous source of food (the wastes derived from fish farming).

Prior to perform the ANOVA, normality was checked by means of Q-Q plots, and the homogeneity of variances was checked using Cochran tests. If significant differences were found after running the model, the post-hoc test SNK was performed (Underwood 1997). When this was the case, we calculated the effect sizes and the confidence intervals at 95% ($CI_{95\%}$) *sensu* Di Stefano (2004) to test whether the statistical significance found was ecologically relevant or not. The rest of the data, when not specified, were reported as mean ± standard error (SE), and statistical tests were performed using a significance level of $\alpha = 0.05$.

Results

$\delta^{13}C$ signatures of fish food used in fish farms were similar (-23.3±0.17‰ and -22.6±0.19‰, respectively) and were markedly lower in the feed than in mussels, which ranged between –21.6±0.18‰ and -19.4±0.08‰. For $\delta^{15}N$ signatures, there was less than one unit of difference between both fish foods (5.3±0.16‰ and 4.5±0.06‰, respectively), while values of the mussels ranged between 5.9±0.10‰ and 4.3±0.06‰ (Fig. 2).

Fish farming did not appear to have a significant effect on the isotopic signatures of C and N of the mussels. In the case of $\delta^{13}C$, mussels both near and far from the fish farms were in the same range (between -22.1‰ and -19.0‰). In the case of $\delta^{15}N$, mussels close to fish farms were in the range of between 6.1‰ and 3.9‰, while references sites were in the range of between 6.4‰ and 3.5‰. The bi-plot of $\delta^{13}C$ and $\delta^{15}N$ signatures in mussels had a similar grouping pattern among locations at both depths. The $\delta^{13}C$ signatures of mussels did not differ significantly between depths,

ranging from -22.1‰ to -19‰, and -21.8‰ to -19‰, for surface and mid-water depths, respectively. For $\delta^{15}N$ signatures, although the ranges were similar at each depth class (from 6.3‰ to 3.5‰, and from 6.4‰ to 3.5‰, for surface and mid-water depth, respectively), the mussels at surface depths had statistically higher levels in comparison to those at mid-water depth ($p<0.05$), with signatures of 4.92±0.09‰ and 4.73±0.10‰, for surface and mid-water depth, respectively (Fig. 2 & table 1).

The $\delta^{13}C$ and $\delta^{15}N$ contents were significantly influenced by the specific locations at which the mussels had grown, and depended on depth (table 1). The mean trophic parameters calculated within locations, such as, CR, NR, CD, NND, SDNND, C and N centroids, were not significantly influenced by fish farming or depth, having similar values for each level (tables 2 & 3).

Discussion

This study suggests that fish farming wastes are not directly assimilated by mussels, and consequently that they do not constitute a major part of their diet, irrespective of the site-specific conditions and depth in the water column.

$\delta^{13}C$ has been widely used as a tracer in trophic studies, allowing inferences about the diet of organisms under a time-integrated basis (Hobson & Welch 1992; Post 2002). In this study, aquaculture wastes did not influence the concentration of $\delta^{13}C$ in mussels, indicating that fish farming wastes constituted a very low percentage of the total diet of the studied individuals, which sides with previous studies (Irisarri *et al.* 2015; Mazzola & Sara 2001; Navarrete-Mier, Sanz-Lazaro & Marin 2010). Even when modelling the consumption of potential food sources, considering only autochthonous particulate organic matter such as fish feed and fish faeces (controversially excluding phytoplankton, their preferred source of food) fish farming wastes were expected to

constitute less than 35% of their diet (Gao *et al.* 2006). Under stratified water conditions, mussel production can be maximized when cultured at optimum depth (Sanz-Lazaro et al. in prep.). However, in this study the $\delta^{13}C$ content in mussels did not seem to be influenced either by depth or by the interaction between aquaculture influence and depth, indicating that at both depths the assimilation of fish farming wastes by mussels was comparably low.

$\delta^{15}N$ generally indicates the trophic level of the species or community (Post 2002). Our results show that fish farming did not have a significant effect on the accumulation of $\delta^{15}N$ in mussels, and consequently did not influence their trophic level. In contrast, $\delta^{15}N$ accumulation in mussels was influenced by depth, although the differences between depths were very small, with values of 4.92±0.18‰ and 4.73±0.18‰ (mean ± $CI_{95\%}$), for surface and mid-water depth respectively. Consequently, this result is not considered to be biologically relevant, because it is generally accepted that changes in trophic level involve modifications of $\delta^{15}N$ levels by around 3‰ (Post 2002).

$\delta^{13}C$ and $\delta^{15}N$ content was notably influenced by the location at which the mussels were taken, and within locations, diet was influenced by depth. This could be explained by the predominance of different natural sources with their respective trophic levels at each location, indicating that mussels can use a range of food sources and that their selectivity may vary depending on availability (Widdows, Fieth & Worrall 1979). Mussels generally feed on phytoplankton, but they can also feed on organic matter particles and nauplii from zooplankton (Davenport, Smith & Packer 2000; Lehane & Davenport 2002; Lehane & Davenport 2004; Molloy *et al.* 2011), all of which may vary depending on the level of eutrophication, and other site-specific conditions, as well as with depth. Our data suggest that among all the foods assimilated by mussels, fish

farming wastes did not seem to be preferred in any situation, and consequently did not constitute a significant part of their diet.

Despite the variation in food sources of mussels at different locations and depths, the trophic niche of all mussel populations indicated very similar trophic structure, diversity and redundancy according to the isotope metrics proposed by Layman et al. (2007). Fish farming did not seem to influence the trophic niche of mussels, which contrasts with the findings of Weldrick & Jelinski (2016). This may be because all of the fish farms that we studied were at a certain distance from the coastline and were in relatively well-flushed areas.

While the theoretical background of IMTA is appealing, its implementation does not seem to be straightforward, and only seems suitable, to some extent, in enclosed areas (Irisarri *et al.* 2014; Weldrick & Jelinski 2016). Nevertheless, the expansion of aquaculture is currently focussed on offshore areas in which there is a lower pressure of cumulative impacts with other activities, and in which the dispersion of fish farm wastes is greater, thus reducing their environmental impact (Holmer 2010). Previous studies using filter-feeding bivalves (mainly mussels) in IMTA (Handa *et al.* 2012a; Irisarri *et al.* 2015; Mazzola & Sara 2001; Navarrete-Mier, Sanz-Lazaro & Marin 2010) appear to agree with the outcomes of this study, indicating that fish farming wastes may constitute only a small fraction of their diet, regardless of site-specific conditions such as the eutrophication level and depth. These results seem to contrast with previous laboratory experiments in which filter-feeding bivalves were shown to assimilate fish farming wastes (Handa *et al.* 2012b; MacDonald, Robinson & Barrington 2011; Redmond *et al.* 2010). This apparent contradiction can be explained by the fact that filter-feeding bivalves have a selective diet and seem to prefer plankton over non-living particles in the water column, as the food quality of the former is greater (Kiorboe, Molenberg &

Nohr 1980). This means that bivalves will feed on fish farming wastes if they are the prevalent food source (MacDonald, Robinson & Barrington 2011), but that in natural conditions they would feed preferentially on other available food sources, mainly phytoplankton but also zooplankton. Our results integrate at least one year of assimilation by mussels; although mussels could assimilate some fish farming wastes during a specific period of the year when nutrients were naturally low (Handa *et al.* 2012a; Irisarri *et al.* 2014), the overall effect throughout the year seems to be negligible.

Filter-feeding bivalves are the main group of low-trophic-level species used in marine IMTA, and within this guild mussels are the most important group (Cranford, Reid & Robinson 2013). Therefore, this experiment used mussels as a model of bivalve filter feeders. However, each specific group or species of bivalve filter feeders can ingest particles of a very specific range, and other groups of filter-feeding bivalves that ingest other specific ranges of particles could give different outcomes. One would expect similar outcomes because filter-feeding bivalves generally have a preference for plankton over non-living particles (Kiorboe, Molenberg & Nohr 1980), and because similar results have been found in other filter-feeding bivalves such as oysters (Navarrete-Mier, Sanz-Lazaro & Marin 2010). Nevertheless, a specific experiment should be done to prove this.

The outcomes of this study do not necessarily demonstrate that IMTA is not suitable, but rather that a greater research effort is needed to achieve the successful implementation of IMTA, which is a promising tool to increase the sustainability of aquaculture. Filter-feeding bivalves seem to be more efficient in confined waters such as ponds (Ferreira, Saurel & Ferreira 2012) and enclosed coastal areas such as narrow inlets (e.g. rías) where currents are weak (Irisarri *et al.* 2014) and, thus, the persistence of particles is high. This greatly limits the use of IMTA based on filter-feeding bivalves

to a relatively small number of the locations in which aquaculture currently occurs, and in which it is expected to expand further (Holmer 2010). One possible solution to increase the assimilation of fish farming wastes would be to use a combination of filter-feeding bivalve species that ingest different ranges of particle size, or to use species that prefer non-living particles (or at least that do not preferentially feed on plankton). Although IMTA has been focused on filter-feeding bivalves, other species such as algae or deposit feeders could be used together in order to obtain synergist results in the assimilation of fish farming wastes by different species types (Cubillo *et al.* 2016). Future research effort should focus in testing the above ideas.

In conclusion, this study demonstrates that mussels have a low assimilative capacity of fish farming wastes, regardless of the specific conditions of the area (such as the level of eutrophication, water stratification, current speed, etc). More research on IMTA should be carried out in order to increase its effectiveness. Most importantly, we propose a shift in the rationale of IMTA, modifying the concept of direct assimilation of wastes to a more global approach of IMTA based on the regional budgets of nutrients in a water body. These issues are crucial to help make aquaculture a more sustainable activity and promote its expansion.

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Table 1: Summary of the ANOVA results on the content of $\delta^{13}C$, $\delta^{15}N$, C and N in mussels (*Mytilus galloprovincialis*). Significant differences are indicated in bold.

		$\delta^{13}C$			$\delta^{15}N$			C			N		
Source of variation	df	MS	*F*	*P*	MS	*F*	*P*	MS	*F*	*P*	MS	*F*	*P*
Aquaculture influence=Aq	1	0.43	0.08	>0.7	0.01	<0.01	>0.9	13.94	0.43	>0.5	0	0	>0.9
Depth=De	1	1.01	2.98	>0.1	1.14	5.21	**<0.05**	9.92	0.67	>0.4	0.09	0.34	>0.5
Location(Aq)	10	5.32	31.26	**<0.001**	5.22	66.29	**<0.001**	32.69	7.46	**<0.001**	2.53	6.56	**<0.001**
AqxDe	1	0.39	1.14	>0.3	0.22	0.99	>0.3	5.08	0.34	>0.5	0.02	0.09	>0.7
Location(Aq)xDe	10	0.34	1.99	**<0.05**	0.22	2.78	**<0.01**	14.91	3.4	**<0.001**	0.27	0.69	>0.7
Residual	96	0.17			0.08			4.38			0.39		
Total	119												
Cochran's *C* test		*C*=0.12, *P*>0.05			*C*=0.12, *P*>0.05			*C*=0.13, *P*>0.05			*C*=0.13, *P*>0.05		

Table 2: Isotope metrics proposed by Layman et al. (2007) of mussels at surface and mid-water depth in the water column under (Aq+) and no (Aq-) influence of aquaculture (mean ± SE, n=6). Each replicate was calculated using the signatures of $\delta^{13}C$ and $\delta^{15}N$ in five mussels, representing a specific population at each location and each depth. Comparisons were made of $\delta^{13}C$ range (CR), $\delta^{15}N$ range (NR), mean distance to centroid (CD), mean nearest neighbour distance (NND) and standard deviation of the nearest neighbour distance (SDNND). For the ecological meaning of each metric see the Materials and Methods section.

		CR	NR	CD	NND	SDNND	C centroid	N centroid
Surface	Aq+	0.97 ± 0.05	0.79 ± 0.09	20.8 ± 0.36	3.65 ± 0.92	7.3 ± 1.74	-20.6 ± 0.36	5.07 ± 0.29
	Aq-	0.87 ± 0.16	0.62 ± 0.14	21 ± 0.34	3.78 ± 0.93	7.18 ± 1.71	-20.3 ± 0.42	5.35 ± 0.31
Mid-water	Aq+	0.92 ± 0.11	0.57 ± 0.11	20.7 ± 0.28	3.64 ± 0.89	7.2 ± 1.73	-20.4 ± 0.29	4.79 ± 0.23
	Aq-	0.87 ± 0.14	0.51 ± 0.09	20.7 ± 0.31	3.77 ± 0.91	7.11 ± 1.71	-20.1 ± 0.4	5.25 ± 0.43

Table 3: Summary of the ANOVA results of the isotope metrics proposed by Layman et al. (2007). See table 2 for an explanation of the acronyms.

		CR			NR			CD			NND			SDNND			C centroid			N centroid		
Source of variation	df	MS	*F*	*P*	MS	*F*	*P*	MS	*F*	*P*	MS	*F*	*P*	MS	*F*	*P*	MS	*F*	*P*	MS	*F*	*P*
Aqua. influence=Aq	1	0.034	0.382	>0.5	0.083	1.153	>0.2	0.1	0.157	>0.6	0.331	2.775	>0.1	0.024	0.205	>0.6	0.077	0.137	>0.7	0.002	0.005	>0.9
Depth=De	1	0.004	0.05	>0.8	0.166	2.307	>0.1	0.309	0.488	>0.4	0	0.003	>0.9	0.032	0.272	>0.6	0.211	0.376	>0.5	0.227	0.418	>0.5
AqxDe	1	0.003	0.039	>0.8	0.02	0.281	>0.6	0.057	0.089	>0.7	0	0	>0.9	0.004	0.034	>0.8	0.089	0.158	>0.6	0.04	0.074	>0.7
Residual	20	0.089			0.072			0.635			0.119			0.116			0.56			0.542		
Total	23																					
Cochran's *C* test		*C*=0.42, *P*>0.05			*C*=0.39, *P*>0.05			*C*=0.31, *P*>0.05			*C*=0.46, *P*>0.05			*C*=0.37, *P*>0.05			*C*=0.32, *P*>0.05			*C*=0.45, *P*>0.05		

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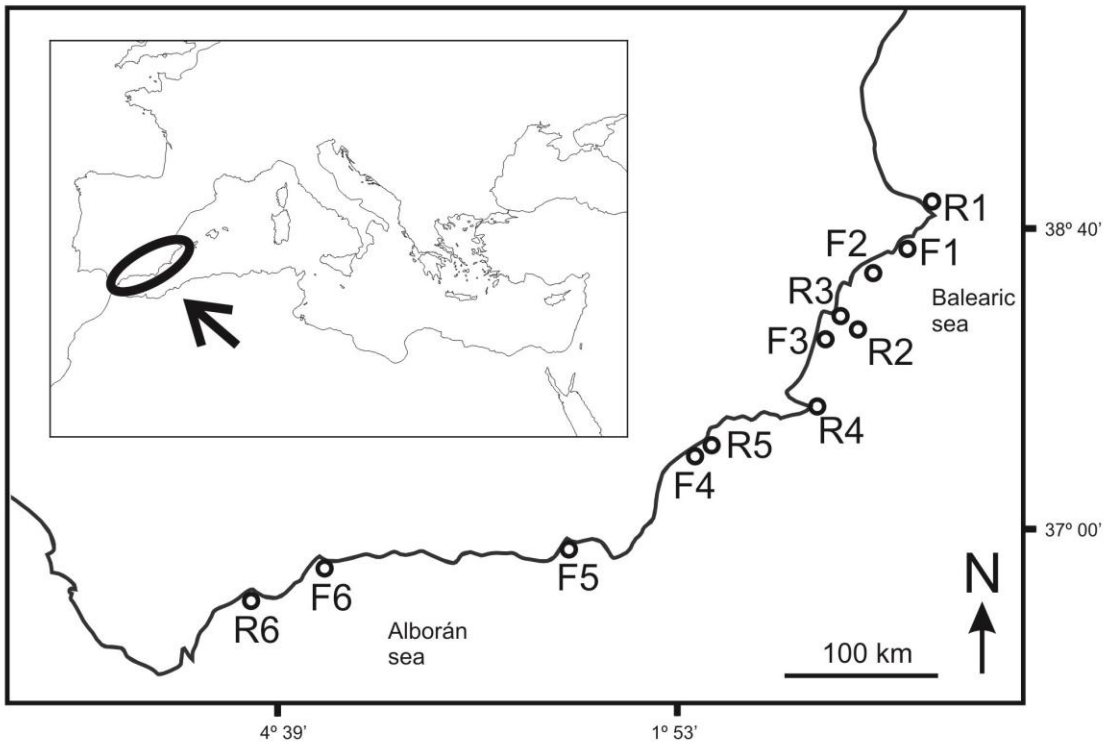
Figure captions

Fig. 1: Map of the locations where mussels for the experiment were sampled. Labels indicate locations influenced ("F") and not influenced by fish farming ("R").

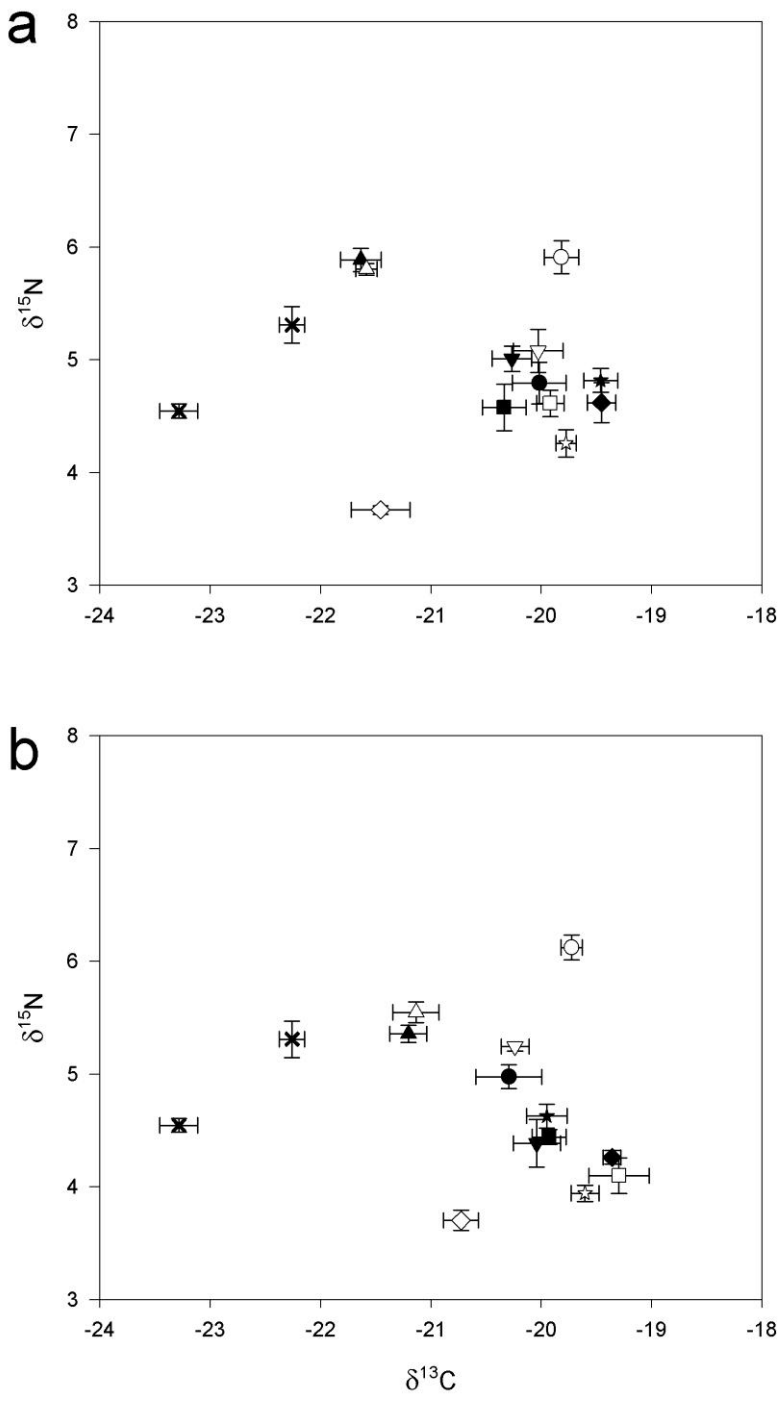
Fig. 2: $\delta^{13}C$ and $\delta^{15}N$ signature in the two types of fish food (×) used in the studied fish farms and in the mussels (n=5, mean ± SE), taken at locations influenced (solid symbols) and not influenced by fish farming (open symbols) corresponding to location 1 (○), 2 (▽), 3 (△), 4 (□), 5(◇) and 6 (☆) (see Table S1 for the positioning of each location) at two depths per location: surface (from 3 to 5 m depth; a) and mid-water (from 12 to 16 m depth; b).

Figures

Fig. 1



586 Fig. 2



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Supplementary material

Table S1: List of the sampling locations at which mussels were sampled along the Mediterranean coast of Spain. See Fig. S1 for the graphical positioning of the locations in the map.

Code of the location	Town (province)	Geographic coordinates
F1	Altea (Alicante)	38º34’18.02’’N 0º02’00.95’’O
F2	Campello (Alicante)	38º25’12.27’’N 0º20’53.08’’O
F3	Guardamar (Alicante)	38º05’08.72’’N 0º35’50.60’’O
F4	Águilas (Murcia)	37º24’46.08’’N 1º32’07.30’’O
F5	Agua dulce (Almería)	36º48’51.50’’N 2º32’12.56’’O
F6	Málaga (Málaga)	36º42’07.01’’N 4º21’35.04’’O
R1	Jávea (Alicante)	38º47’52.68’’N 0º11’33.55’’E
R2	Alicante (Alicante)	38º08’31’’N 0º25’50.11’’O
R3	Alicante (Alicante)	38º10’41.03’’N 0º29’43.35’’O
R4	Cabo de Palos (Murcia)	37º37’29.70’’N 0º40’42.80’’O
R5	Águilas (Murcia)	37º25’31.56’’N 1º30’33.69’’O
R6	Marbella (Málaga)	36º30’15.63’’N 4º52’05.13’’O

Figure S1. Chlorophyll a concentration ($mg \cdot m^{-3}$; log scale) in the study area estimated monthly with a resolution of 4 km from 30-09-2010 to 01-10-2016 using the Moderate Resolution Imaging Spectroradiometer (MODIS) integrated in the Aqua satellite of NASA. Data was adquired from the Giovanni web page (https://giovanni.gsfc.nasa.gov/giovanni/) from NASA.